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TIEG1 Inhibits Breast Cancer Invasion and Metastasis by Inhibition of Epidermal Growth Factor Receptor (EGFR) Transcription and the EGFR Signaling Pathway

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TIEG1 can induce apoptosis of cancer cells, but its role in inhibiting invasion and metastasis has not been reported and is unclear. In this study, we find that decreased TIEG1 expression is associated with increased human epidermal growth factor receptor (EGFR) expression in breast cancer tissues and cell lines. TIEG1 plays an important role in suppressing transcription of *EGFR* by directly binding to the *EGFR* promoter. While overexpression of TIEG1 attenuates *EGFR* expression, knockdown of TIEG1 stimulates *EGFR* expression. Furthermore, TIEG1 and HDAC1 form a complex, which binds to Sp1 sites on the *EGFR* promoter and inhibits its transcription by suppressing histone acetylation. TIEG1 significantly inhibits breast cancer cell invasion, suppresses mammary tumorigenesis in xenografts in mice, and decreases lung metastasis by inhibition of *EGFR* gene transcription and the EGFR signaling pathway. Therefore, TIEG1 is an antimetastasis gene product; regulation of *EGFR* expression by TIEG1 may be part of an integral signaling pathway that determines and explains breast cancer invasion and metastasis.

Human epidermal growth factor receptor (EGFR) plays a critical role in the signal transduction pathway for cell proliferation, apoptosis, angiogenesis, and metastasis (11, 37). Overexpression of *EGFR* is found in approximately 30% of human primary tumors and has been significantly associated with disease stage, prognosis, survival, and response to chemotherapy (4, 20). EGFR is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases: EGFR, HER2/c-neu, Her3 (ErbB-3), and Her4 (ErbB-4) (1, 27). It is the first transmembrane receptor tyrosine kinase that has been cloned and sequenced and can be activated by binding to its specific ligands, including epidermal growth factor (EGF) and transforming growth factor α (TGF-α) (39).

EGFR has been shown to be quite important in breast cancer. *EGFR* expression predicts BRCA1 status in patients with breast cancer (35). Levels of *EGFR* are significantly elevated in women with breast cancer compared with control levels, and increased *EGFR* levels may be an early marker of breast cancer (25). Breast cancer patients with tumors positive for *EGFR* expression have a less favorable prognosis than those with tumors negative for *EGFR* expression. However, for those patients whose tumors have been tested and found to be *EGFR* positive, blocking *EGFR* expression has been shown to reduce risk of breast cancer in general (2, 22).

The 5′-regulatory sequence of the *EGFR* gene contains a GC-rich promoter, which is located in direct proximity to one enhancer element. Basal transcription of the *EGFR* gene is regulated by the transcription factor Sp1 (3, 16). Previous *in vitro* and *in vivo* studies showed that a common polymorphism in the *EGFR* promoter region is associated with altered promoter activity and gene expression, and in order for *EGFR* promoter activity to occur, it has been discovered that multiple Sp1 binding sites are required (21). Another study demonstrates that the *EGFR* promoter can be transactivated by wild-type and tumor-derived mutant p53 (9, 23). Other data also strongly suggest that the *EGFR* promoter is regulated by retinoic acid receptor γ (RAR-γ), which itself is under the control of retinoic acid (RA) (40). *EGFR* is also a target gene transcriptionally activated by Stat5b and downregulated by CPEB3 in neurons (24). However, the detailed regulation of EGFR in humans is complicated and remains largely unknown. TGF-β inducible early gene 1 (TIEG1) is a transcription factor, which can bind to Sp1 sites on many gene promoters and regulate their transcription; two Sp1 sites were found to exist on the *EGFR* promoter region by bioinformatic analysis (1, 18, 31). It is also reported that EGFR expression is significantly increased, but TIEG1 expression is lower in breast tumors than in normal breast tissues (4, 28). These two clues indicate that TIEG1 might play an important role in regulating EGFR transcription. The aim of the present study was to explore the potential role of TIEG1 in the regulation of *EGFR* transcription and to reveal the role of TIEG1 involved in EGFR-mediated invasion and metastasis of breast cancer. Our studies are helpful in demonstrating the epigenetic modification of the *EGFR* promoter induced by TIEG1 and in providing a potential target for treatment of EGFR-related breast cancers.

MATERIALS AND METHODS

Patient materials. Ninety pairs of fresh-frozen sporadic breast tumors and their adjacent normal breast tissues were randomly selected from the

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pathology archives and tumor bank of the Cancer Hospital, Fudan University. The informed consent forms (ICF) were obtained in advance from the Institutional Review Board (IRB) of the Cancer Hospital, Fudan University. The tumor specimens were all invasive ductal carcinomas, according to WHO tumor classification.

Cell lines, culture, plasmids, and transfection. Human breast cancer cells MCF-7, MDA-MB-231, and MDA-MB-468 were purchased from ATCC (American Type Culture Collection, Manassas, VA). The highly metastatic (HM) MDA-MB-231HM cell line was established by our institute. MCF-7 and MDA-MB-468 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. MDA-MB-231 and MDA-MB-231HM cells were routinely maintained in Leibovitz's L-15 medium with 2 mM L-glutamine at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO_2). The medium was supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The medium was changed every 2 to 3 days, and cells were subcultured by treatment with 0.25% trypsin–0.53 mM EDTA (EDTA) solution. Cells were checked routinely and found to be free of contamination by *Mycoplasma* or fungi. All the cell lines were discarded after 3 months, and new lines were obtained from frozen stock.

The TIEG1 overexpression vector with an amino-terminal FLAG epitope tag (pcDNA4/TO-TIEG) and its control empty vector (pcDNA4/TO) were kindly provided by Malayannan Subramaniam and Thomas C. Spelsberg of the Department of Biochemistry and Molecular Biology at the Mayo Clinic and Foundation (Rochester, MN). The EGFR expression vector (pCMV6-EGFR) and control vector were purchased from Origene Company (Rockville, MD). An EGFR promoter/luciferase construct, pGL3-EGFR, was generated by cloning the 292-bp KpnI-NheI fragment (bp −316 to −25, oriented to the ATG translation start site as position +1) of the 5′ region of the *EGFR* gene into the luciferase expression vector pGL3B by using primers as described previously (12). pGL3-EGFRmut was generated from pGL3-EGFR, in which the two Sp1 sites (−147 to −142 and −111 to −106) were mutated from CCGCCC to CCGTTC.

Briefly, for transient transfection, cells were seeded in six-well plates at a density of 4×10^5 cells/well. The following day, cells were transfected with the indicated expression vector for 8 h. Following transfection, cells were maintained in medium plus 10% FBS for the times indicated in Results and the figure legends.

Reverse transcription-PCR (RT-PCR). Total RNA was extracted from cells or tissue with TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA). The reverse transcription reaction was performed using a Superscript First-Strand Synthesis System (Invitrogen Life Technologies, Inc., Carlsbad, CA). The newly synthesized cDNA was amplified by PCR. The reaction mixture contained 2 μl of cDNA template, 1.5 mM $MgCl_2$, 2.5 units of *Taq* polymerase, and 0.5 μM primer; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer was used as an internal control.

RNA extraction and real-time PCR. Total RNA was extracted from cells or tissue with TRIzol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA) and quantified by UV absorbance spectroscopy. Real-time quantitative PCR was performed using a fluorescence temperature cycler (Opticon; MJ Research) and a SYBR green PCR Core Reagents kit according to the manufacturer's instructions (Takara, Dalian, China). The relevant primers were as follows: EGFR primers 5′-TCCCTCAGCCACCCA TATGTAC-3′ and 5′-GTCTCGGGCCATTTTGGAGAATTC-3′, TIEG1 primers 5′-ACTGCGGAGGAAAGAATGGA-3′ and 5′-CTGGGAGGAG TGCTGGGAAC-3′, and GAPDH primers 5′-GCCAAAAGGGTCATCA TCTC-3′ and 5′-GTAGAGGCAGGGATGATGTTC-3′. An initial incubation of 50°C for 2 min was followed by denaturing at 95°C for 10 s and then 40 cycles at 95°C for 15 s and 60°C for 1 min. PCR products were detected by bound SYBR green double-stranded DNA fluorescence, and the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method was used to enable quantification of the mRNA of these genes. All samples were tested in triplicate. Target gene expression was compared to that of the housekeeping gene, GAPDH. After PCR, a melting curve was obtained and analyzed.

Immunohistochemical detection of TIEG1 and EGFR. We studied surgical biopsy specimens from a set of 90 female breast cancer patients whose breast cancer tissues were analyzed for TIEG1 and EGFR expression. Tumor sections were subjected to immunohistochemical staining for TIEG1 and EGFR. Tissue sections were incubated with monoclonal antibodies against human TIEG1 or EGFR (Santa Cruz, Biotechnology, Santa Cruz, CA) at a dilution of 1:100 overnight at 4°C. The ABC peroxidase method of staining was then employed as described by the manufacturer (ABC; Vector Laboratories, Burlingame, CA). The slides were colorimetrically detected with 3,3′-diaminobenzidine (DAB). For TIEG1 or EGFR, the reaction was considered positive if more than 10% of the cells showed distinctive nuclear staining.

Western blot analysis. Cells were washed twice with phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride and lysed in mammalian protein extraction buffer (Pierce, Rockford, IL). The lysates were transferred to Eppendorf tubes and clarified by centrifugation at 12,000 × *g* for 40 min at 4°C. Identical amounts (50 μg of protein) of cell lysates were resolved by SDS-PAGE, and the proteins were transferred to nitrocellulose. The membranes were incubated in blocking solution consisting of 5% powered milk in PBS plus 0.1% Tween 20 (PBST) at room temperature for 1 h, followed by immunoblotting with the following: EGFR antibody, TIEG1 antibody, vascular endothelial growth factor (VEGF) antibody, and metalloproteinase 9 (MMP-9) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); extracellular signal-regulated kinase (t-ERK) antibody and phospho-ERK (p-ERK) antibody (Cell Signaling Technology, Danvers, MA); or GAPDH antibody (Sigma-Aldrich, St. Louis, MO). Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

siRNA preparation and transfection. The small interfering RNA (siRNA) targeting TIEG1 and nontargeting siRNA were purchased from Shanghai GeneChem (Zhangjiang, Shanghai). The target sequence for TIEG1 is 5′-GCTAAATGACATTGCTCTACC-3′. The cells in the exponential phase of growth were seeded in six-well plates at a concentration of 5×10^5 cells/well. After incubation for 24 h, the cells were transfected with siRNA specific for TIEG1 and nontargeting siRNA at a final concentration of 100 nM. Silencing was examined at 48 h after transfection.

ChIP and ChIP-reChIP assays. Chromatin immunoprecipitation (ChIP) assays were carried out according to the manufacturer's protocol (Active Motif, Carlsbad, CA). Briefly, cells in 150-mm tissue culture dishes were fixed with 1% formaldehyde and incubated for 10 min at 37°C. The cells were then washed twice with ice-cold PBS, harvested, and resuspended in ice-cold TNT lysis buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1% aprotinin). The lysates were sonicated to shear the DNA to fragments of 200 to 600 bp and subjected to immunoprecipitation with the following antibodies: TIEG1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), acetylated histone H3 ([Ac-H3] Abcam Inc., Cambridge, MA), or IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as a negative control. Three micrograms of antibody was used for each immunoprecipitation. The antibody-protein complexes were collected by protein G beads and washed three times with ChIP wash buffer (5% SDS, 1 mM EDTA, 0.5% bovine serum albumin, and 40 mM $NaHPO_4$, pH 7.2). The immune complexes were eluted with 1% SDS and 1 M $NaHCO_3$, and the cross-links were reversed by incubation at 65°C for 4 h in the presence of 200 mM NaCl and RNase A. The samples were then treated with proteinase K for 2 h, and DNA was purified by minicolumn, ethanol precipitation and resuspended in 100 ml of H_2O. The primers corresponding to the *EGFR* promoter region, −316 and −25 upstream of the transcription start site (sense, 5′-GCCTGGTCCCTCCTCCTC-3′; antisense, 5′-CGGC TCTCCCGATCAATAC-3′), were used for real-time PCR or PCR to detect the presence of the EGFR promoter DNA. As negative controls, we tested for the recruitment of TIEG1 or histone deacetylase 1 (HDAC1) at

exon 19 of the EGFR gene using the primers (sense) 5′-GCAATATCAGC CTTAGGTGCGGCTC-3′ and (antisense) 5′-CATAGAAAGTGAACATT TAGGATGTG-3′.

For ChIP-reChIP, briefly, after sonication, chromatin was incubated overnight with 5 μg of TIEG1 antibody or IgG as negative control. After several washings, the beads were incubated with 50 μl of buffer containing 0.5% SDS and 0.1 M $NaHCO_3$ for 10 min at 65°C. The supernatant was collected after spinning, diluted with 1 mM EDTA, 150 mM NaCl, 50 mM HEPES (pH 7.5), 0.1% SDS, 1% Triton X-100, and 0.1% sodium deoxycholate, and incubated with 3 μg of the HDAC1 antibody (Millipore Corp., Billerica, MA) overnight. After samples were washed, protein-DNA complexes were eluted from beads and treated with proteinase K overnight. DNA was purified with a mini-column, and the DNA binding to the EGFR endogenous promoter was quantified by real-time PCR or PCR using the primers described above.

Luciferase reporter gene assay. In luciferase reporter assays, MDA-MB-231 cells were seeded in six-well plates at a density of 1×10^5 to 2×10^5 cells/well and cultured for 24 h. Cells were then cotransfected with the EGFR promoter/luciferase construct (0.5 μg/well) with 0.5 μg of control vector or TIEG1 expression vector, together with 20 ng of control the *Renilla* luciferase reporter construct pRL-TK (Promega, Madison, WI). The total amount of DNA per well was adjusted to 1.5 μg by the addition of sonicated salmon sperm DNA. Luciferase assays were performed as recommended by the vendor (Promega, Madison, WI) and normalized relative to protein concentration as determined by a bicinchoninic acid protein assay (Pierce, Rockford, IL). The promoter activity was then expressed as luminescence units, which is the ratio of luminescence counts of cell lysate to the absorbance at 595 nm for the same amount of cell lysate stained with bicinchoninic acid protein assay reagent.

Cell invasion assay. Invasion experiments were conducted with a Matrigel invasion chamber (BD Labware). Each upper chamber was coated with Matrigel. A total of 10^5 cells was added to the top of this upper chamber. The chamber was then transferred to a well containing 500 μl of medium containing 10% FBS. Cells were incubated at 37°C for 36 h. Cells in the top well were removed by wiping the top of the membrane with cotton swabs. The filters were fixed in 10% formalin and stained with hematoxylin and eosin (H&E), and the remaining cells were counted under a light microscope at a magnification of ×200. Five fields were counted for each sample.

Tumorigenicity and metastasis assays in athymic mice. Female athymic BALB/c *nu*/*nu* mice, 4 to 6 weeks old, were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Science. All studies on mice were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Shanghai Medical Experimental Animal Care Committee. Animals were divided into five groups: MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, MDA-MB-231, MDA-MB-468/TIEG1, and MDA-MB-468/Vector. Each group had 10 mice. Cells (1.5×10^6 cells) were injected into the mammary fat pad. Animals were monitored every 2 days for tumor growth and general health. The rate of primary tumor growth of different cells was determined by plotting the means of two orthogonal diameters of the tumors, measured at 7-day intervals. Animals were sacrificed and autopsied at 6 weeks after cell inoculation. At the end of the experiment, trunk blood was collected, and the serum was separated and analyzed by enzyme-linked immunosorbent assay (ELISA) for VEGF and MMP-9 expression according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN). The lungs were used to evaluate the level of metastases and fixed in Bouin's solution for 24 h and then stored in 100% ethanol. When the lungs were restored their inherent color, the white metastatic deposits could be assessed by macroscopic observation. To confirm the presence of lung metastases, sections were cut at 50-μm intervals, and H&E staining was performed. In this study, the number of metastatic nodules on the lung surface was counted. Two independent pathologists calculated the number of metastases.

Tumor tissues were fixed with 10% formaldehyde for 24 h and subsequently embedded with paraffin. Then microvessel density (MVD) was detected and quantified as previously reported (15). Microangiography for blood vessels was performed using beamline BL13W1, an X-ray imaging and biomedical application station of the Shanghai Synchrotron Radiation Facility (SSRF) in China. The maximum light size of the beam was 45 mm (horizontal) by 5 mm (vertical) at the object position at 20 keV. All animals were anesthetized by intraperitoneal injection of ketamine (200 mg/kg) (Ketanest; Pfizer, Karlsruhe, Germany). The image contrast agent barium sulfate, suspended in glycerol (50% water solution; a concentration of 0.5 g/ml), was injected into the left ventricle. Serial images of tumor blood vessels in nude mice were then recorded at the SSRF.

Statistical analysis. Association between TIEG1 and *EGFR* expression was assessed using Pearson's χ^2 test. Analysis of variance and Student's *t* test were used to determine the statistical significance of differences between experimental groups. *P* values of less than 0.05 were considered significant, and the confidence intervals quoted were at the 95% level. Statistical analysis was performed using SPSS software, version 13.0 (SPSS, Chicago, IL).

RESULTS

TIEG1 expression is associated with *EGFR* expression and metastasis in breast tumors. To study the relationship between TIEG1 and *EGFR*, slices of tumor samples from 90 patients were tested for TIEG1 or EGFR with immunohistochemistry. As shown in Fig. 1A, we found TIEG1 expression was absent or lower in breast cancer tissues, while EGFR expression was remarkably higher in breast cancer tissues. TIEG1 and EGFR gene expression were also analyzed in breast cancer tissues by real-time PCR. As shown in Fig. 1B, results of TIEG1 and *EGFR* mRNA levels were similar to the results of immunohistochemical detection of TIEG1 and EGFR shown in Fig. 1A. A strong correlation was found between TIEG1 expression and *EGFR* expression in breast tumors ($P < 0.001$) (Table 1).

To study the relationship between TIEG1 and metastasis in clinic samples, the staining grades of TIEG1 were analyzed in relation to lymph node metastasis conditions of patients. Our results revealed a significantly positive association between TIEG1 expression and lymph node metastasis ($P < 0.001$) (Table 2).

In order to study the mechanism underlying the regulation of *EGFR* by TIEG1, we chose four cell lines for the following *in vitro* studies, MCF-7, MDA-MB-468, MDA-MB-231 parental cell line, and MDA-MB-231HM with high pulmonary metastatic potential. In our experiments, we found EGFR protein levels were low in MCF-7 and MDA-MB-231 cells but very high in MDA-MB-468 and MDA-MB-231HM cells, while TIEG1 protein levels were high in MCF-7 and MDA-MB-231 cells but very low in MDA-MB-468 and MDA-MB-231HM cells (Fig. 1C).

Binding status of TIEG1 complex and histone acetylation on the EGFR promoter between MDA-MB-231 and MDA-MB-231HM cells. In our experiments, we found that the *EGFR* mRNA level was low in MDA-MB-231 cells and very high in MDA-MB-231HM cells, while the TIEG1 mRNA level was high in MDA-MB-231 cells and very low in MDA-MB-231HM cells (Fig. 2A, B, and C).

To determine if TIEG1 or HDAC1 could bind to the EGFR promoter, MDA-MB-231 and MDA-MB-231HM cells were subjected to ChIP assays. ChIP results demonstrated that TIEG1 and HDAC1 could bind significantly to the silent EGFR promoter in MDA-MB-231 cells (Fig. 2D and E). Control experiments indicated that the coincubation of cross-linked chromatin with preimmune IgG did not generate a corresponding EGFR amplifica-

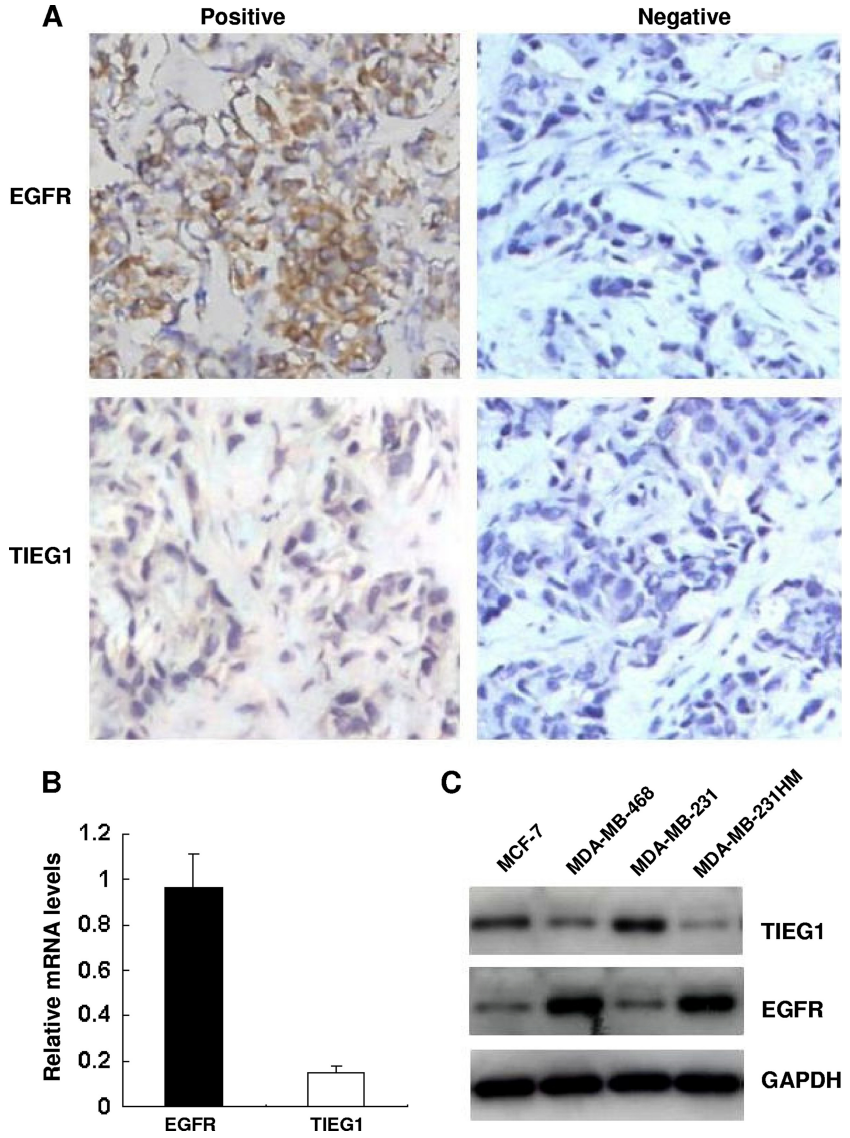

FIG 1 TIEG1 is associated with *EGFR* expression in breast cancer tissues. (A) TIEG1 and EGFR expression in breast cancer tissues. Immunohistochemical staining was carried out on histological sections in 90 pairs of breast cancer tissues with the anti-TIEG1 or anti-EGFR antibody. (B) Relative *TIEG1* and *EGFR* mRNA levels were detected by real-time quantitative PCR in 60 breast cancer tissues. (C) Relative TIEG1 and EGFR protein levels were detected by Western blotting in MCF-7, MDA-MB-468, MDA-MB-231, and MDA-MB-231HM cells.

tion product. These transcriptional factors could not be recruited to the coding region of exon 19 in the EGFR gene (Fig. 2D).

To demonstrate whether TIEG1 is recruited to the *EGFR* promoter through interaction with HDAC1, we performed ChIP-reChIP assays. Cross-linked and fragmented chromatin was pre-

TABLE 1 TIEG1 and EGFR protein expression are positively associated

EGFR expression	TIEG1 expression (no. of samples)[a]	
	Positive	Negative
Positive	8	45
Negative	25	12

[a] Breast cancer tissue specimens from 90 patients were stained with anti-TIEG1 or anti-EGFR antibody.

TABLE 2 TIEG1 expression in tissues of breast cancer with and without lymph node metastasis

Pathological tissue type	n[a]	Grade of TIEG1 expression[b]				Positive rate (%)
		−	+	++	+++	
With lymph node metastasis	57	53	4	0	0	7
Without lymph node metastasis	33	9	18	6	0	73

[a] n, number of samples.

[b] −, no canary color; +, canary color; ++, brown color; +++, cytoplasm and nuclei filled with brown-black color.

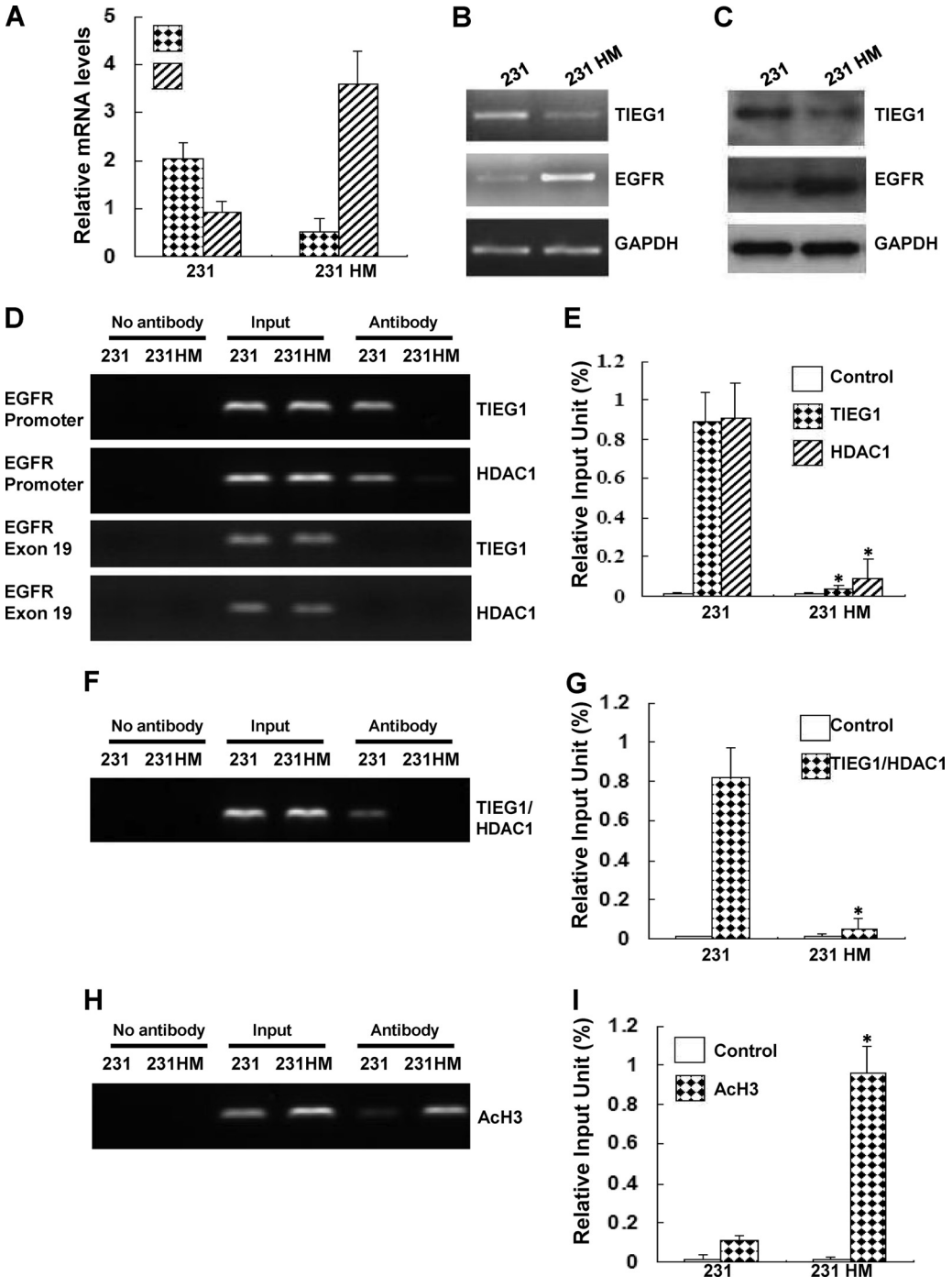

FIG 2 Binding status of TIEG1 complex and histone acetylation on the *EGFR* promoter in MDA-MB-231 and MDA-MB-231HM cells. (A and B) Relative *TIEG1* and *EGFR* mRNA levels were detected in MDA-MB-231 and MDA-MB-231HM cells. Total RNA was extracted from the cells and the real-time PCR (A) or RT-PCR (B) was performed. *, $P < 0.01$ versus controls. (C) Relative TIEG1 and EGFR protein levels are detected in MDA-MB-231 and MDA-MB-231HM cells. Cell lysates were prepared, and equal amounts (50 μg) of proteins were resolved by 6% SDS-PAGE. Proteins were transferred to nitrocellulose membrane, and TIEG1 and EGFR were detected by immunoblotting with monoclonal anti-TIEG1 or anti-EGFR antibody, respectively. GAPDH was used as a loading control. (D) Binding status of TIEG1 or HDAC1 on the *EGFR* promoter or exon 19 of the EGFR gene in MDA-MB-231 and MDA-MB-231HM cells. ChIP assays were performed by using PCR as described in Materials and Methods. (E) Binding status of TIEG1 or HDAC1 on the *EGFR* promoter in MDA-MB-231 and MDA-MB-231HM cells. ChIP assays were performed by using real-time PCR as described in Materials and Methods. *, $P < 0.01$. (F and G) Detection of TIEG1 and HDAC1 complex on the *EGFR* promoter in MDA-MB-231 and MDA-MB-231HM cells. A ChIP-reChIP assay was performed, and chromatin was incubated with TIEG1 antibody and then immunoprecipitated with HDAC1 antibody. The *EGFR* promoter DNA bound to TIEG1/HDAC1 was amplified by PCR (F) or real-time PCR (G). *, $P < 0.01$ versus controls. (H and I) Histone acetylation on the *EGFR* promoter in MDA-MB-231 and MDA-MB-231HM cells. ChIP assay was performed by PCR (H) or real-time PCR (I) as described in Materials and Methods. *, $P < 0.01$ versus controls. 231: MDA-MB-231 cells; 231 HM, MDA-MB-231HM cells.

pared from MDA-MB-231 cells or MDA-MB-231HM cells and sequentially subjected to the first-step ChIP with TIEG1 antibody and the second-step IP with HDAC1 antibody. As shown in Fig. 2F and G, the two-step ChIP-reChIP successfully precipitated the EGFR promoter in MDA-MB-231 cells, indicating that TIEG1 and HDAC1 formed an inhibiting transcriptional complex on the silent EGFR promoter, which might explain the reason for the differential expression of EGFR in these cell lines.

Since HDAC1 is a histone deacetyltransferase and can inhibit histone acetylation, we then investigated the status of histone acetylation on the *EGFR* promoter. ChIP with antibody against acetyl-H3 (Ac-H3) was performed between MDA-MB-231 and MDA-MB-231HM cells. As shown in Fig. 2H and I, a significant increase of Ac-H3 was found on the EGFR promoter in MDA-MB-231HM cells.

In the above experiments, binding of the TIEG1/HDAC1 complex might lead to a lower level of histone acetylation on the silent *EGFR* promoter in MDA-MB-231 cells than in MDA-MB-231HM cells. This indicates that TIEG1 might recruit HDAC1 to silence the *EGFR* promoter, which might play a role in inhibiting *EGFR* transcription.

Overexpression of TIEG1 attenuates *EGFR* expression and histone acetylation on the *EGFR* promoter. To further test the effect of TIEG1 on *EGFR* expression, we overexpressed TIEG1 in MDA-MB-231HM cells, which express low levels of endogenous TIEG1 (Fig. 2B). A TIEG1 expression vector or control vector was transfected into MDA-MB-231HM cells, which generated stable transfectants, MDA-MB-231HM/TIEG1 and MDA-MB-231HM/Vector. RT-PCR and Western blot analysis demonstrated that overexpression of TIEG1 reduced the mRNA and protein level of EGFR in MDA-MB-231HM/TIEG1 cells (Fig. 3A and B).

In ChIP experiments, we found that TIEG1 and HDAC1 could bind to the *EGFR* promoter in MDA-MB-231HM/TIEG1 cells (Fig. 3C and D). In ChIP-reChIP experiments, we also found that TIEG1 could recruit HDAC1 to the *EGFR* promoter in MDA-MB-231HM/TIEG1 cells (Fig. 3E and F). Next, we investigated histone acetylation status on the *EGFR* promoter influenced by TIEG1. ChIP experiments showed that TIEG1 could attenuate acetylation of H3 on the *EGFR* promoter in MDA-MB-231HM/TIEG1 cells (Fig. 3G and H). These results indicated that overexpression of TIEG1 could increase the binding of TIEG1/HDAC1 complex to the *EGFR* promoter and decrease the level of histone acetylation on the *EGFR* promoter in MDA-MB-231HM/TIEG1 cells.

Knockdown of TIEG1 increases *EGFR* expression and histone acetylation on the *EGFR* promoter. To modulate endogenous TIEG1, we treated MDA-MB-231 cells with 100 nM TIEG1 siRNA or nontargeting siRNA for 48 h and performed real-time PCR and Western blot analysis. As shown in Fig. 4A and B, TIEG1 siRNA inhibited TIEG1 mRNA and protein significantly in MDA-MB-231 cells after transfection for 48 h. At the same time, we found that knockdown of TIEG1 increased *EGFR* mRNA and protein significantly.

ChIP results demonstrated that knockdown of TIEG1 decreased the binding of TIEG1 and HDAC1 to the *EGFR* promoter (Fig. 4C and D). ChIP-reChIP assays demonstrated that knockdown of TIEG1 decreased the recruitment of TIEG1 and HDAC1 complex on the *EGFR* promoter (Fig. 4E and F). ChIP results demonstrated that knockdown of TIEG1 increased histone H3 acetylation on the *EGFR* promoter (Fig. 4G and H).

Effect of TIEG1 on the *EGFR* promoter activity. To further identify the role of TIEG1 in regulating *EGFR* promoter transcription, we cotransfected the *EGFR* promoter/luciferase construct with TIEG1 or HDAC1 expression vector in MDA-MB-231HM cells and MDA-MB-468 cells and detected *EGFR* promoter activity. Figure 5B and C show that the luciferase activity was inhibited by TIEG1 or HDAC1 in both MDA-MB-231HM cells and MDA-MB-468 cells, further indicating that the TIEG1/HDAC1 complex could inhibit *EGFR* promoter activity.

We also found two Sp1 sites from bp −200 to +1 on the *EGFR* promoter (Fig. 5A). To determine the potential roles of these Sp1 elements in regulation of EGFR gene transcription, TIEG1 was cotransfected with either a wild-type (pGL3-EGFR) or an Sp1 site mutant (pGL3-EGFRmut) EGFR reporter construct into MDA-MB-231HM cells and MDA-MB-468 cells. As shown in Fig. 5D and E, any single mutation of either of the two Sp1 sites increased the reporter gene activity compared with the wild-type *EGFR* promoter construct when EGFR was cotransfected with TIEG1. Mutations of both Sp1 sites simultaneously caused a further increase of reporter gene activity. These results suggested that both Sp1 sites contributed in a concerted mechanism to the TIEG1-inhibited transcription of the EGFR gene and that TIEG1 could inhibit *EGFR* transcription by binding to Sp1 sites on the *EGFR* promoter.

TIEG1 inhibits breast cancer cell invasion by inhibition of the EGFR signaling pathway. To investigate whether expression of TIEG1 could modulate the EGFR signaling pathway, we analyzed some protein levels related to the EGFR signaling pathway between MDA-MB-231HM/TIEG1 and MDA-MB-231HM/Vector cells and between MDA-MB-468/TIEG1 and MDA-MB-468/Vector cells. As shown in Fig. 6A, expression of TIEG1 could significantly inhibit protein levels of EGFR, p-ERK, p-AKT, VEGF, and MMP-9 in MDA-MB-231HM/TIEG1 and MDA-MB-468/TIEG1 cells compared with control cells. Then, we assessed the invasion between MDA-MB-231HM/TIEG1 and MDA-MB-231HM/Vector cells and between MDA-MB-468/TIEG1 and MDA-MB-468/Vector cells. As shown in Fig. 6C and D, TIEG1-expressing cells had a significantly lower invasion potential than control cells; however, overexpression of EGFR restored invaded cells back to the levels seen with MDA-MB-231HM/Vector or MDA-MB-468/Vector cells.

To investigate whether knockdown of TIEG1 could modulate the EGFR signaling pathway, we analyzed some protein levels related to the EGFR signaling pathway in TIEG1-knockdown MDA-MB-231 or MCF-7 cells and their control cells. As shown in Fig. 6B, knockdown of TIEG1 could significantly increase protein levels of EGFR, p-ERK, p-AKT, VEGF, and MMP-9 in TIEG1-knockdown MDA-MB-231 or MCF-7 cells compared with control cells. Then, we assessed the invasion assay in TIEG1-knockdown MDA-MB-231 or MCF-7 cells and their control cells. As shown in Fig. 6E and F, TIEG1-knockdown cells had a significantly higher invasion potential than control cells.

TIEG1 inhibits breast cancer metastasis *in vivo*. In the following experiments, we assessed the effect of TIEG1 on tumor growth and metastasis by using an orthotopic xenograft tumor model in athymic mice. MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, MDA-MB-231, MDA-MB-468/TIEG1, and MDA-MB-468/Vector cells were injected into the mammary fat pad of athymic mice and analyzed for tumor growth at the time intervals indicated in Fig. 7A and B. Results revealed that the tumors in mice bearing MDA-MB-231HM/TIEG1 or MDA-MB-468/TIEG1 cells grew more slowly than the tumors in mice bear-

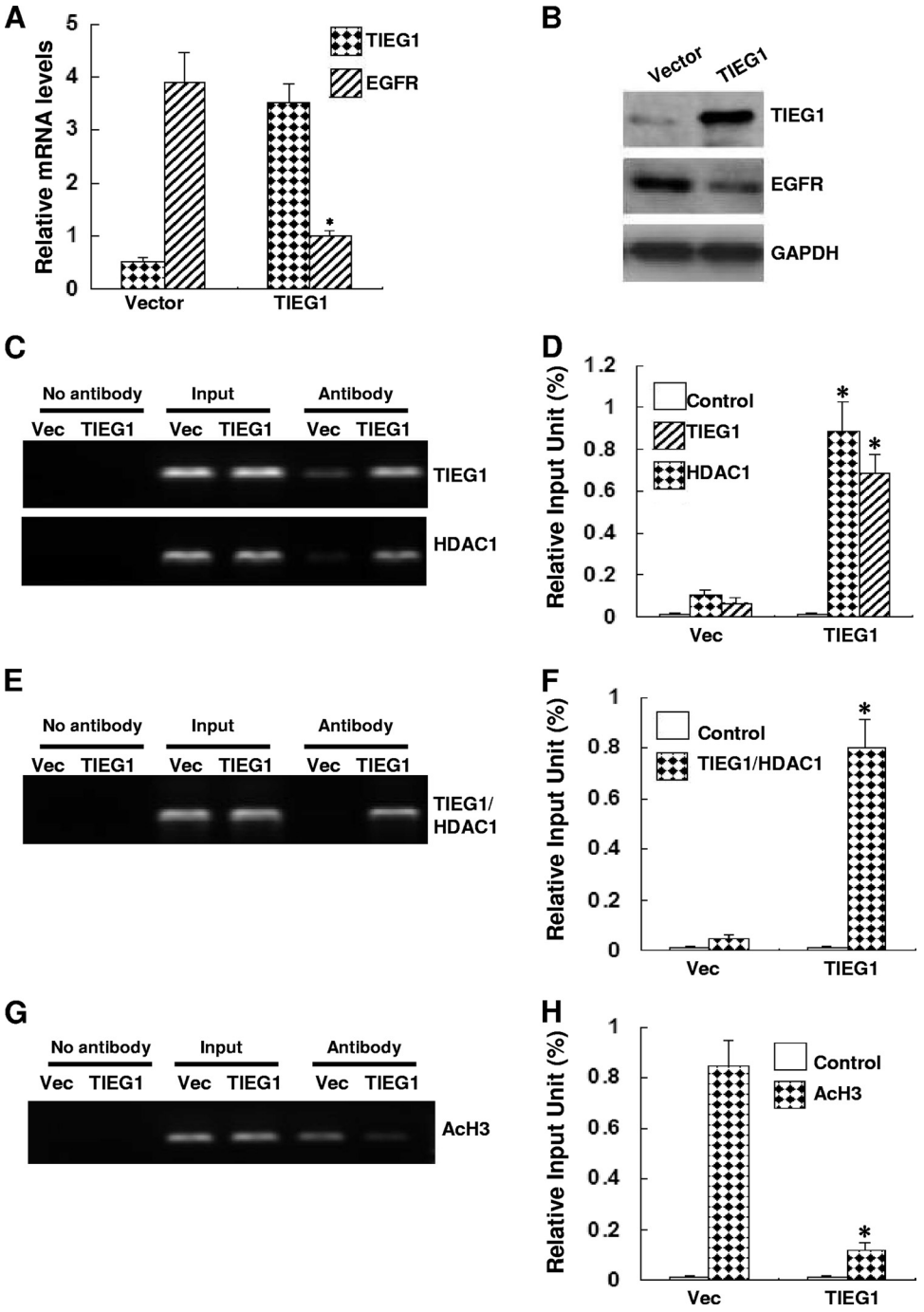

FIG 3 Overexpression of TIEG1 attenuates *EGFR* expression and histone acetylation of the *EGFR* promoter in MDA-MB-231HM cells. (A and B) TIEG1 expression vector or control vector was transfected into MDA-MB-231HM cells and generated stable transfectants, MDA-MB-231HM/TIEG1 and MDA-MB-231HM/Vector. Real-time PCR (A) and Western blot analysis (B) were performed between MDA-MB-231HM/Vector and MDA-MB-231HM/TIEG1 cells. *, $P < 0.01$ versus controls. (C and D) Binding of TIEG1 or HDAC1 on the *EGFR* promoter in MDA-MB-231HM/Vector and MDA-MB-231HM/TIEG1 cells. ChIP assays were performed by using PCR (C) or real-time PCR (D) as described in Materials and Methods. *, $P < 0.01$ versus controls. (E and F) Detection of TIEG1 and HDAC1 complex on the *EGFR* promoter. MDA-MB-231HM cells were treated as described for panels A and B. A ChIP-reChIP assay was performed by using PCR (E) or real-time PCR (F) as described in Materials and Methods. *, $P < 0.01$ versus controls. (G and H) Histone acetylation on the *EGFR* promoter in MDA-MB-231HM/Vector and MDA-MB-231HM/TIEG1 cells. A ChIP assay was performed by using PCR (G) or real-time PCR (H) as described in Materials and Methods. *, $P < 0.01$ versus controls. Vec, MDA-MB-231HM/Vector cells; TIEG1, MDA-MB-231HM/TIEG1 cells.

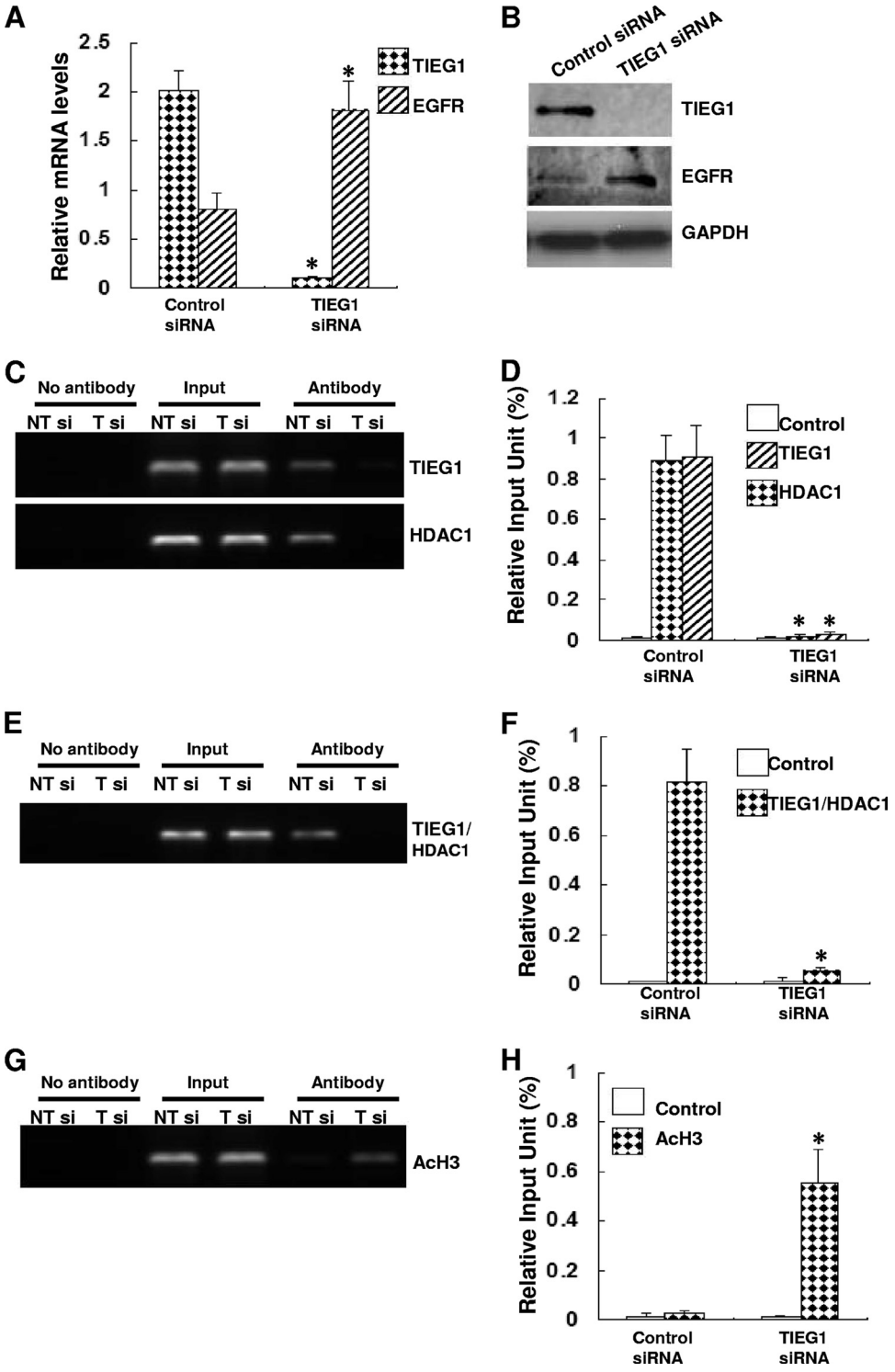

FIG 4 Knockdown of TIEG1 induces *EGFR* expression in MDA-MB-231 cells. (A and B) Knockdown of TIEG1 in MDA-MB-231 cells induced *EGFR* expression. MDA-MB-231 cells were treated with 100 nM TIEG1 siRNA or nontargeting siRNA for 48 h. Real-time PCR (A) and Western blot analysis (B) were performed as described in Materials and Methods. *, $P < 0.01$ versus controls. (C and D) Binding status of TIEG1 or HDAC1 on the *EGFR* promoter in MDA-MB-231 cells transfected with TIEG1 siRNA or nontargeting siRNA. Cells were treated as described for panel A. ChIP assays were performed by using PCR (C) or real-time PCR (D) as described in Materials and Methods. *, $P < 0.01$ versus controls. (E and F) Binding status of TIEG1/HDAC1 on the *EGFR* promoter in MDA-MB-231 cells transfected with TIEG1 siRNA or nontargeting siRNA. Cells were treated as described for panel A. A ChIP-reChIP assay was performed by using PCR (E) or real-time PCR (F) as described in Materials and Methods. *, $P < 0.01$ versus controls. (G and H) Histone acetylation on the *EGFR* promoter in MDA-MB-231 cells transfected with TIEG1 siRNA or nontargeting siRNA. Cells were treated as described for panel A. ChIP assays were performed by using PCR (G) or real-time PCR (H) as described in Materials and Methods. *, $P < 0.01$ versus controls. NT si or Control siRNA, MDA-MB-231 cells transfected with nontargeting siRNA; T si or TIEG1 siRNA, MDA-MB-231 cells transfected with TIEG1 siRNA.

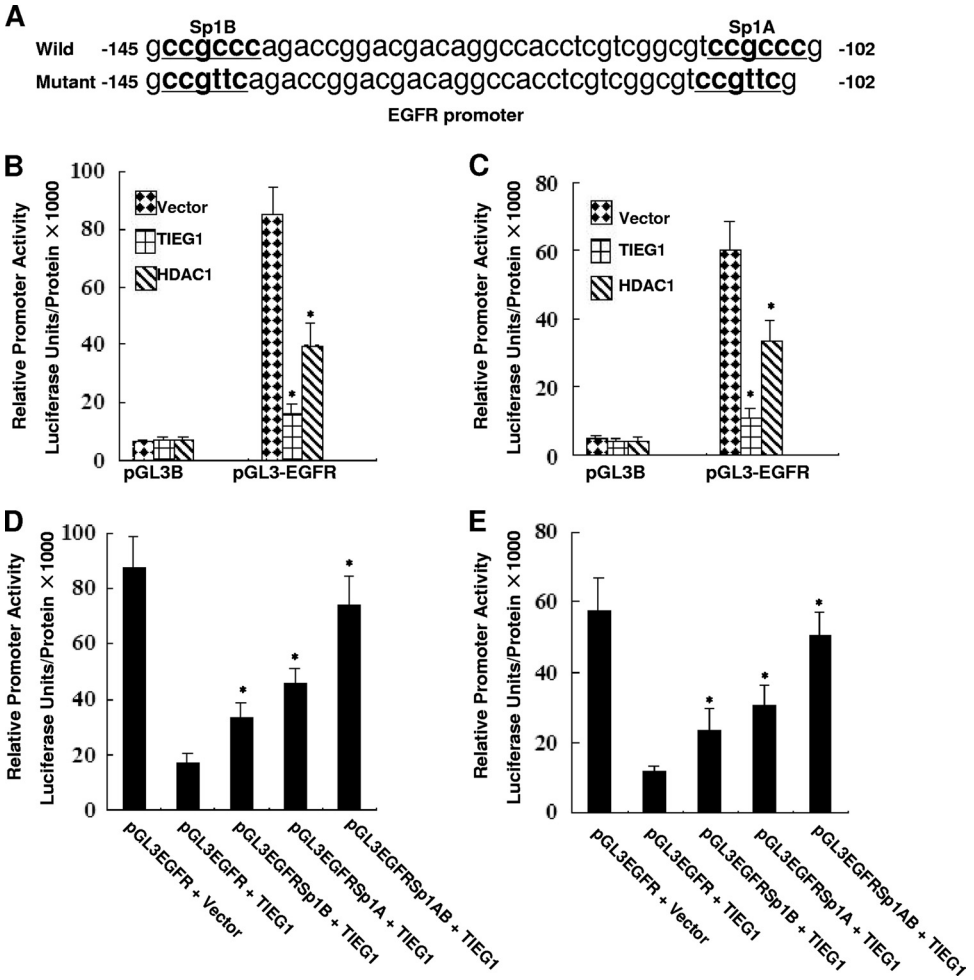

FIG 5 TIEG1 inhibits *EGFR* promoter activity in MDA-MB-231HM and MDA-MB-468 cells. (A) Model depicting binding of TIEG1 transcriptional factor to the Sp1 sites on the *EGFR* promoter. (B and C) TIEG1 and HDAC1 inhibited *EGFR* promoter activity in MDA-MB-231HM and MDA-MB-468 cells. MDA-MB-231HM (B) or MDA-MB-468 (C) cells were plated in six-well tissue culture plates and then cotransfected with 0.5 μg of pGL3-EGFR with 0.5 μg of TIEG1 or HDAC1 expression vector or control vector for 48 h; luciferase activity was detected as described in Materials and Methods. *, $P < 0.05$ versus controls ($n = 3$ experiments). (D and E) TIEG1 requires two intact Sp1 sites for *EGFR* promoter inhibition. A wild-type (pGL3-EGFR) or mutant (pGL3-EGFRmut) EGFR reporter construct (0.5 μg) was transfected into MDA-MB-231HM (D) or MDA-MB-468 cells (E) with or without 0.5 μg of TIEG1 expression vector for 48 h. Luciferase activity was detected as described in Materials and Methods. *, $P < 0.05$ versus controls ($n = 3$ experiments).

ing MDA-MB-231HM/Vector or MDA-MB-468/Vector cells (Fig. 7A and B). In addition, we measured the amounts of VEGF and MMP-9 in the mouse serum by ELISA. The levels of VEGF and MMP-9 were significantly lower in the serum from mice bearing MDA-MB-231HM/TIEG1 and MDA-MB-468/TIEG1 cells than in control groups (Fig. 6C and D).

MVD of tumor sections from mice bearing MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, and MDA-MB-231 cells was determined. As shown in Fig. 7E and G, MVD of tumor sections decreased significantly in tumors expressing high levels of TIEG1 relative to levels in the control groups.

Blood vessels in the tumors of mice bearing MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, and MDA-MB-231 cells were quantified as well. As shown in Fig. 7F and H, the number of blood vessels in tumors decreased significantly in tumors expressing high levels of TIEG1 relative to levels in control groups.

To study pulmonary metastasis influenced by TIEG1, lungs from mice bearing MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, and MDA-MB-231 cells were examined physically at autopsy and then subjected to microscopic examination for morphological evidence of tumor cells by light microscopy on H&E-stained paraffin sections. The incidence of lung metastasis decreased significantly in mice bearing MDA-MB-231HM/TIEG1 cells compared with mice bearing control MDA-MB-231HM/Vector cells (Fig. 7I and J).

DISCUSSION

TIEG1 encodes a three-zinc-finger Krüppel-like transcription factor, which is originally cloned from human osteoblasts (OBs) as a primary response gene following TGF-β treatment (30). TIEG1 mimics TGF-β action and plays a role in inducing apoptosis in human osteoblast cells, pancreatic carcinoma cells, and epithelial and liver cancer cells (5, 29, 32, 33). We previously reported that TIEG1 expression was significantly upregulated by homoharringtonine and Velcade and that TIEG1 is a key regulator which

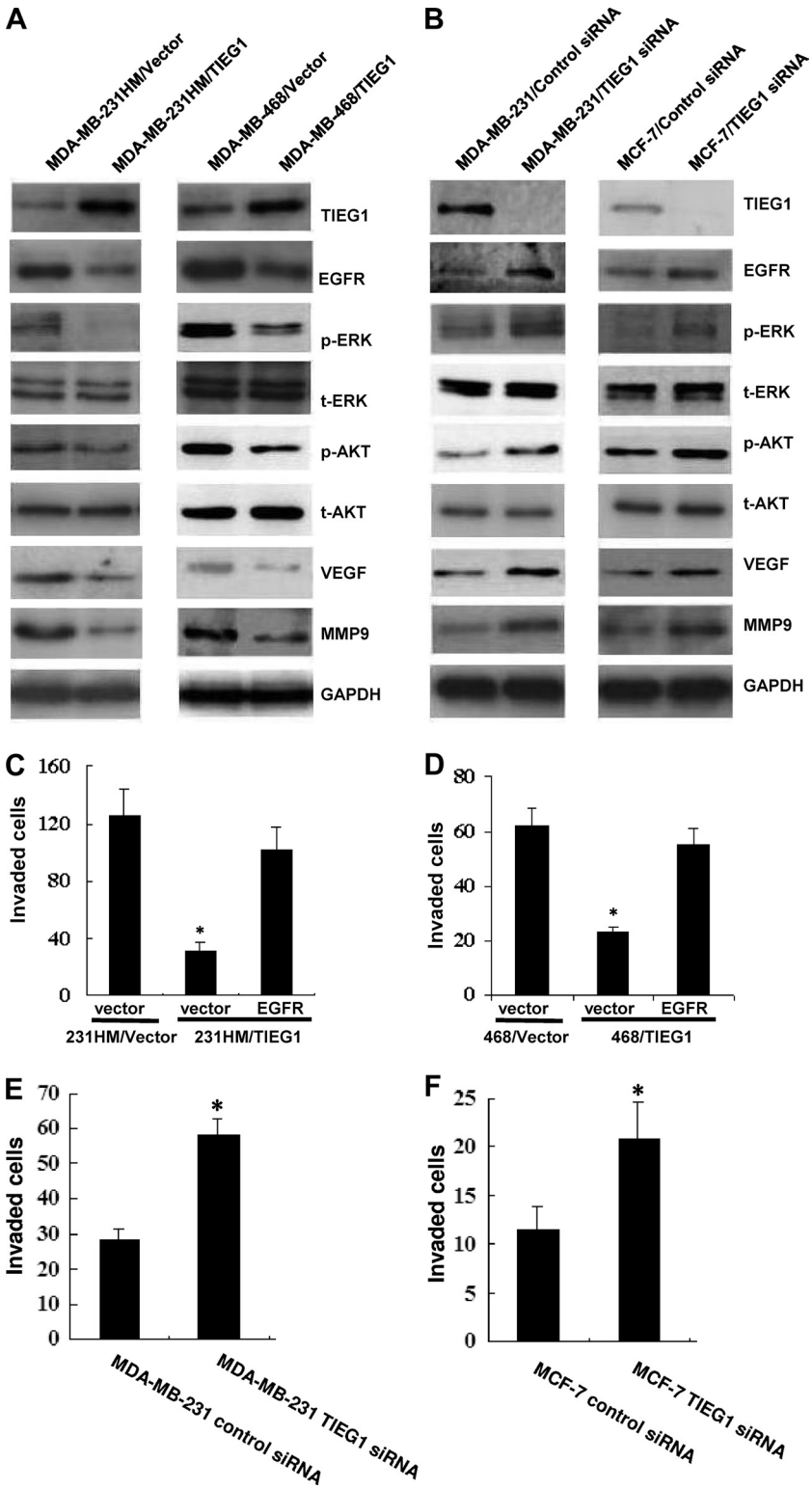

FIG 6 TIEG1 inhibits breast cancer cell invasion by inhibition of EGFR signaling pathway. (A) Overexpression of TIEG1 inhibited the EGFR signaling pathway. TIEG1 expression vector or control vector was transfected into MDA-MB-231HM or MDA-MB-468 cells and generated stable transfectants, MDA-MB-231HM/TIEG1 and MDA-MB-231HM/Vector or MDA-MB-468/TIEG1 and MDA-MB-468/Vector, respectively. Western blot analysis was performed as described in Materials and Methods. (B) Knockdown of TIEG1 induced the EGFR signaling pathway. MDA-MB-231 or MCF-7 cells were treated with 100 nM TIEG1 siRNA or nontargeting siRNA for 48 h. Western blot analysis was performed as described in Materials and Methods. (C and D) Expression of TIEG1 inhibited breast cancer cell invasion; overexpression of EGFR restored levels of invaded cells. MDA-MB-231HM/TIEG1 (231HM/TIEG1), MDA-MB-231HM/Vector (231HM/Vector), MDA-MB-468/TIEG1 (468/TIEG1), and MDA-MB-468/Vector (468/Vector) cells, as indicated, were transfected with 2 μg of EGFR expression vector or control vector and analyzed for Matrigel invasion. *, $P < 0.01$ versus controls. (E and F) Knockdown of TIEG1 promoted cancer cell invasion. MDA-MB-231 cells or MCF-7 cells transfected with nontargeting siRNA (control siRNA) or TIEG1-directed siRNA were analyzed for Matrigel invasion and Western blotting. *, $P < 0.01$ versus controls. Values in panels C to F indicate invaded cells/mean numbers in five microscope fields.

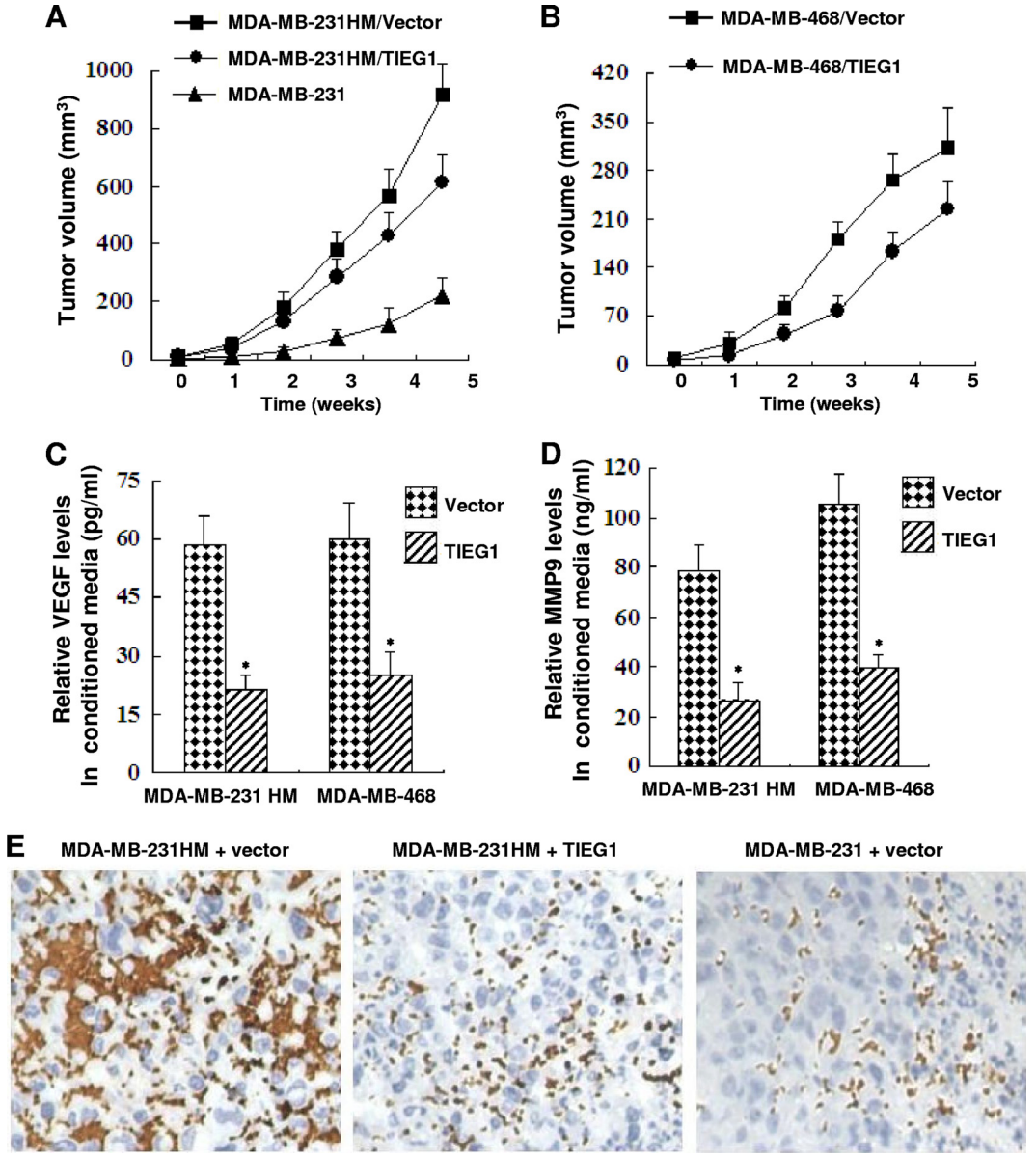

FIG 7 TIEG1 inhibits breast cancer metastasis by inhibition of EGFR signaling pathway. (A and B) MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, MDA-MB-231, MDA-MB-468/TIEG1, and MDA-MB-468/Vector cells, as indicated, were injected into the mammary fat pad of athymic mice and analyzed for tumor growth at the indicated time intervals. (C and D) The amounts of VEGF and MMP-9 in the serum of mice bearing MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, MDA-MB-468/TIEG1, or MDA-MB-468/Vector cells were detected by ELISA. *, $P < 0.05$ versus controls. (E) Tumor sections with CD34 antibody staining for MVD (original magnification, ×200) from mice bearing MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, and MDA-MB-231 cells. (F) Blood vessels in the mice bearing MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, and MDA-MB-231 cells were detected by a microangiographic technique at SSRF. (G) Quantification of microvessel density of tumor sections from mice bearing MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, and MDA-MB-231 cells. *, $P < 0.01$ versus controls. (H) Quantification of blood vessels of tumors from mice bearing MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, and MDA-MB-231 cells. *, $P < 0.01$ versus controls. Values are per mm^3. (I) Photomicrographs of micrometastases (arrow) in lung sections obtained from mice bearing MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, and MDA-MB-231 cells (H&E staining; magnification, × 200). (J) Metastasis numbers per lung (per average numbers from 10 mouse lungs) obtained from mice bearing MDA-MB-231HM/TIEG1, MDA-MB-231HM/Vector, and MDA-MB-231 cells. *, $P < 0.01$ versus controls.

can induce and promote apoptosis through the mitochondrial apoptotic pathway (17). TIEG1 can bind to Sp1 sites on the osteoprotegerin (OPG), Samd7, and M-myc promoters and regulate their transcription (1, 18, 31). In this paper, bioinformatic analysis of the 5′ flanking region of the human *EGFR* gene showed that there existed two Sp1 sites (CCGCCC) in the *EGFR* promoter region from −147 to −142 and −111 to −106, which indicated that TIEG1 might bind to the *EGFR* promoter and regulate *EGFR* transcription. However, the role of TIEG1 in regulating *EGFR* expression has not been previously reported and remained unclear. In this study, we investigated the activity and mechanism of TIEG1 in inhibiting EGFR.

We found that TIEG1 expression was nearly absent and that *EGFR* expression was markedly increased in random-select breast

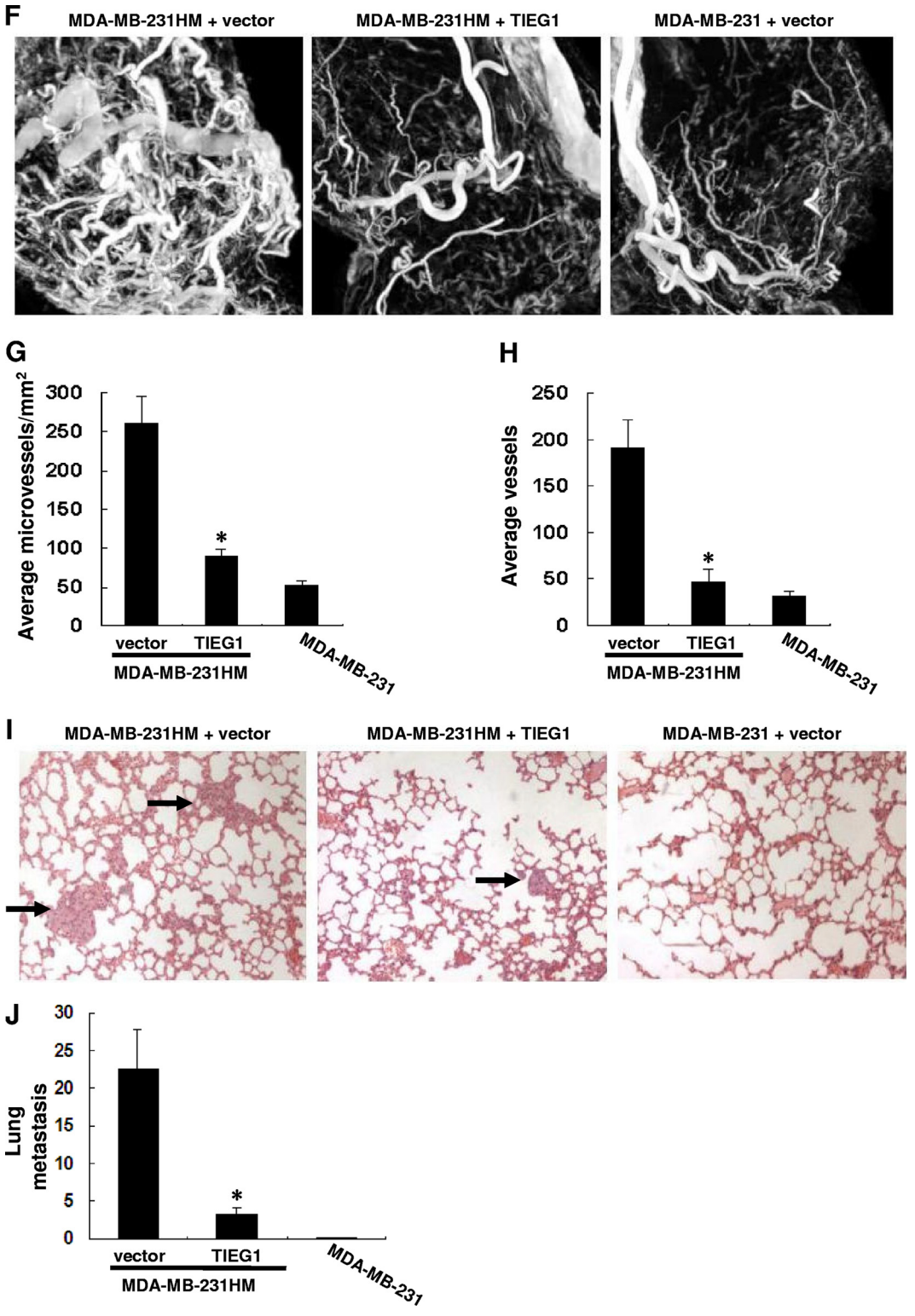

FIG 7—*Continued.*

tumors. This strong correlation was found between TIEG1 and *EGFR* expression in breast cancer tissues and four breast cancer cell lines, indicating that TIEG1 might play an important role in regulating *EGFR* transcription.

We initially established MDA-MB-231HM cells with high pulmonary metastatic potential in our laboratory (6, 10). Then, we found that TIEG1 expression was reduced and that *EGFR* expression was increased in the highly metastatic MDA-MB-231HM

cells compared with its parental MDA-MB-231 cells. All these data further confirmed the potential association between TIEG1 and EGFR.

In our studies, we found that TIEG1 inhibited *EGFR* transcription. However, point mutation of Sp1 sites in the *EGFR* promoter construct abrogated the inhibitory effect of TIEG1 on *EGFR* promoter activity, indicating that TIEG1 inhibited *EGFR* promoter activity by directly binding to the Sp1 sites of the *EGFR* promoter.

TIEG1 is known to associate with corepressor mSin3A to recruit histone deacetylase, and it is well known that mSin3A interacts with histone deacetylases 1 and 2 and forms a corepressor complex (7, 14, 38). In our studies, we found that TIEG1 and HDAC1 formed an inhibitory transcriptional complex on the silencing *EGFR* promoter in MDA-MB-231 cells. HDAC1 is an enzyme that influences transcription by selectively deacetylating the core histone proteins (13). Our experiments showed that histone H3 was heavily acetylated on the *EGFR* promoter in MDA-MB-231HM cells, which was the result of lower binding activity of TIEG1/HDAC1 on the *EGFR* promoter than in MDA-MB-231 cells.

Based on the above experiments, we hypothesized that TIEG1-induced epigenetic changes involving histone modifications, chromatin remodeling, and transcription were responsible for *EGFR* silencing. To further confirm the roles of TIEG1 and its related transcriptional factors in regulating *EGFR* transcription, we overexpressed TIEG1 in MDA-MB-231HM cells and detected the binding status of the TIEG1/HDAC1 complex and histone acetylation on the *EGFR* promoter during this process.

In these experiments, we found that overexpression of TIEG1 in MDA-MB-231HM cells attenuated *EGFR* expression. ChIP experiments demonstrated that the TIEG1/HDAC1 complex could bind to the *EGFR* promoter in TIEG1-overexpressing MDA-MB-231HM cells. Overexpression of TIEG1 attenuated acetylation of H3 on the *EGFR* promoter. The results indicated that overexpression of TIEG1 recruited more HDAC1 to the *EGFR* promoter and induced epigenetic characteristics on the *EGFR* promoter.

We also found that knockdown of TIEG1 could increase *EGFR* expression by inhibiting the recruitment of the TIEG1/HDAC1 complex and increasing histone acetylation on the *EGFR* promoter region in MDA-MB-231 cells, which further confirmed that TIEG1-induced epigenetic changes involving histone modifications and chromatin remodeling were responsible for *EGFR* expression.

In this study, we found that decreased TIEG1 expression was associated with lymph node metastasis in breast tumors in the clinic. TIEG1-expressing cells showed significantly lower invasion potential than control cells, while TIEG1-knockdown cells had significantly higher invasion potential. TIEG1 could inhibit orthotopic tumor growth, tumor microvessel density, angiogenesis, and pulmonary metastasis significantly *in vivo*.

EGFR activation triggers multiple signal transduction pathways, including the Ras/Raf/mitogen-activated protein kinase (ERK) pathway and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (19, 34, 36). EGFR inhibition can decrease VEGF expression and, consequently, angiogenesis in many tumor types (26). EGFR activation promotes disruption of adherens junctions through induction of MMP-9 (8). Our previous study showed that *Pseudomonas aeruginosa* mannose-sensitive hemagglutinin (PA-MSHA) could significantly inhibit protein levels of EGFR, p-ERK, p-AKT, VEGF, and MMP-9, while also inhibiting the metastatic potential of breast cancer cells by inhibition of the EGFR signaling pathway (22). In this study, we again found that TIEG1 could inhibit protein levels of EGFR, p-ERK, p-AKT, VEGF, and MMP-9. All these data indicate that TIEG1 inhibited invasion, angiogenesis, and metastasis of breast cancer cells by inhibition of the EGFR signaling pathway.

We conclude that TIEG1 expression is associated with *EGFR* expression in breast tumors and cell lines. TIEG1 plays an important role in inhibiting *EGFR* promoter activity by directly binding to the *EGFR* promoter. Overexpression of TIEG1 attenuates *EGFR* expression, while knockdown of TIEG1 stimulates *EGFR* expression. TIEG1 interacts with HDAC1, forming an inhibitory transcriptional complex which binds to Sp1 sites on the *EGFR* promoter and prevents its transcription by inhibiting histone acetylation. TIEG1 significantly inhibits invasion, angiogenesis, and metastasis of breast cancer cells by inhibition of *EGFR* gene transcription and the EGFR signaling pathway. Therefore, TIEG1 is an antimetastasis gene product, and regulation of *EGFR* expression by TIEG1 may be part of an integral signaling pathway that determines and explains breast cancer invasion and metastasis. Detecting the status of TIEG1 expression in patients may help to identify the onset of breast cancer metastasis.

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We declare that we have no conflicts of interest.

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